

## Inhibitors of Electron Flow: Photosynthesis

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Inhibitor studies have become imperative in view of the intricate structure of the chloroplast and the complex mechanisms involved in the photosynthetic process. Despite extensive research in different areas of photosynthesis, mystery still surrounds certain areas of electron carriers, particularly those located between the two photosystems. The positions and functions of cytochromes Cyt b 559, low and high potential, and Cyt b 563 are not established as yet<sup>1</sup>. None of the components participating on the electron donating side of PSII has been clearly defined, though several participants are strongly indicated<sup>1</sup>. The importance of electron inhibitor studies with regard to photosynthetic electron flow thus stems from the fact that it helps to (i) locate the position of the electron carriers between the two photosystems; (ii) elucidate the sequence of the electron carriers; (iii) unravel the mechanisms involved in the energy conservation process; and (iv) describe the role of each electron carrier in relation to the energy conservation process.

Since several electron inhibitors are particularly found to interfere with the electron flow between the two photosystems, an inhibitory class of plastoquinone antagonists was introduced in 1970-71 with the investigation of halogenated or hydroxylated lipophilic benzoquinones<sup>2</sup>. In 1944, Warburg and Lutgens<sup>3</sup> demonstrated the photoreduction of *p*-benzoquinones by chloroplasts and the present inhibitor studies involving the use of halogenated or hydroxylated lipophilic benzoquinones are obviously what may be called as a follow up research of Warburg's effort in the right direction. Whereas alkyl substituted *p*-benzoquinones are photoreduced, halogen substituted benzoquinones appear to be inhibitors of photosynthetic electron transport<sup>4</sup>.

In fact the last two decades witnessed a remarkable application of inhibitors in an effort to understand the mechanism involved in photosynthetic electron transport and in the allied process leading to the development of ATP molecules and NADPH which together are known as "assimilatory power"<sup>5</sup> (Arnon) or "Photoreducing equivalents" or "reducing power"

(Levine and Norman) and are required for the fixation of CO<sub>2</sub> in the dark phase of photosynthesis (Calvin cycle).

Earlier, Groinet-Elhanan<sup>6</sup> reported antimycin A and heptylhydroxyquinone oxide to have had inhibited cyclic photophosphorylation driven by photosystem I. In continuation of these studies, numerous compounds have been synthesized which affect the cyclic or noncyclic photophosphorylations. Warburg and Lutgens<sup>3</sup>, Bishop<sup>7</sup>, Trebst and Eck<sup>8</sup>, Tagawa *et al.*<sup>9</sup>, Bamberger *et al.*<sup>10</sup>, Izawa *et al.*<sup>11</sup>, Steihl and Witt<sup>12</sup>, Drechsler *et al.*<sup>13</sup> and Zweig *et al.*<sup>14</sup> studied in detail the mechanism involved in antimycin A inhibitions and the effect of numerous substituted benzoquinones as acceptors of Hill reactions. Of paramount importance is the conclusion arrived at by Zweig *et al.*<sup>14</sup>, using dichloronaphthoquinone, that the site of inhibition is located close to plastoquinone (PQ) in the down Hill electron chain. Steihl and Witt<sup>12</sup> indicated that in the down Hill electron chain, PQ may be the primary electron acceptor of PSII. Based on the above clues, in 1961 Trebst and Eck<sup>8</sup> attempted to inhibit the electron flow by applying an entirely new type of inhibitor, dibromothymoquine, and ultimately succeeded in inhibiting electron flow at the reducing side of plastoquinone. Further investigations by Bohme *et al.*<sup>15</sup> and several other workers revealed that 2, 5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) is the best inhibitor. Ever since Bishop investigated the role of plastoquinone in petroleum ether extracted chloroplasts, a method first introduced by Bishop himself, attempts were made to establish a relationship between the inhibitors and plastoquinone. In 1971, Bohme *et al.*<sup>15</sup> ultimately documented that DBMIB is the true antagonist of PQ and that exogenous addition of PQ reverses DBMIB inhibition.

Mention may also be made here of 3, 4-dichlorophenyl-1, 1-dimethyl urea (DCMU), a weed killer or a herbicide, which was extensively used as a potential inhibitor of photosynthetic electron flow prior to the synthesis of DBMIB.

Inhibition of photosynthetic electron flow could be achieved either by mutagenic techniques or by

introducing chemical inhibitors. Employing mutagenic techniques, Levine *et al.*<sup>16-22</sup> made remarkable contributions to inhibitor studies involving screening of photosynthetic mutants. They conducted a series of experiments on *Chlamydomonas reinhardtii*.

Today inhibitor studies have come to stay in the field of photosynthesis chiefly because of their extensive applicability and the systematic approach involved in them. Reports from various parts of the world today indicate the extensive application of innumerable inhibitors, including DBMIB and DCMU in the research of photosynthesis<sup>23-42</sup>. However, a greater part of the credit goes to Trebst *et al.* and Levine *et al.* for their pioneering research and impressive findings.

### Mutagenic Inhibitor Techniques

The production of photosynthetic mutants has added a new dimension to electron flow inhibitor studies<sup>43</sup>. In 1949, Davis<sup>44</sup> reported for the first time the isolation and characterization of several photosynthetic mutants of the green alga, *Chlorella*. The term 'photosynthetic mutant' is intended to differentiate between the more frequently occurring 'pigment mutant' and those altered specifically through the mechanism of photosynthesis. Sagar and Zalokar<sup>45</sup> and Chance and Sagar<sup>46</sup> isolated numerous pigment mutants from *Chlamydomonas* and *Chlorella* and studied the light induced oxidation of various components of the cell; they concluded that the *Chlamydomonas* mutant strain which suffered from a strongly decreased ratio of  $\beta$ -carotene/chlorophyll performed a more than adequate photosynthesis on chlorophyll a basis<sup>46</sup>. This represents one of the few cases where a pigment mutant has been successfully used in direct studies on photosynthesis<sup>43</sup>. In 1958, Bishop<sup>43</sup> studied the manner in which oxygen was developing during photosynthesis using strain *D*<sub>3</sub> of the alga *Scenedesmus obliquus*, which had undergone biochemical mutations. This alga has adaptable hydrogenase which under appropriate conditions allows photoassimilation of CO<sub>2</sub> and hydrogen gas without the production of oxygen that normally occurs in photosynthesis<sup>43</sup>. These studies were further extended by several other scientists and a conclusion was drawn about the possible applicability of photosynthetic mutants. Whereas some mutants could photoreduce CO<sub>2</sub> during the dark phase and evolve O<sub>2</sub>, some mutants could still photoreduce during the dark phase but were not able to generate O<sub>2</sub>. Recent detailed studies revealed that some mutants which exhibit specific deviations and changes in the EPR signals could be obtained and be conveniently used for studying the photochemistry of photosynthesis and for separating the oxygen evolving system<sup>47</sup>.

Further, mutations affecting partial reactions of photosynthesis in the green alga *Chlamydomonas reinhardtii* have been a useful technique for the elucidation of photosynthetic mechanisms in green plants<sup>48, 49</sup>. This is based on the fact that when photosynthetic electron transport is blocked, there may be an increase in the level of fluorescence similar to that obtained in the presence of an inhibitor of photosynthetic electron transport, such as DCMU<sup>21</sup>.

#### (1) Mutations Inducing Loss of Electron Transport Carriers

In Table 1 are given various mutant species of *Chlamydomonas*, *Scenedesmus* and *Oenothera* along with the scientists who worked on them and the affected components due to mutations they were subjected to. Levine and Gorman<sup>18-20</sup> conducted experiments using mutant strains of the unicellular green alga *Chlamydomonas reinhardtii* that had lost the capacity to carry out normal photosynthetic electron transport. Light absorbance changes were investigated in chloroplast fragments of wild type *Chlamydomonas reinhardtii* and five different mutant strains, ac-21, ac-206, ac-208, ac-115, ac-141, having impaired photosynthesis. Two absorbance changes of significant nature were observed. One component exhibits maximum absorption at 553 nm and the other at 559 nm. The component exhibiting the 553 nm change is a cytochrome similar to cytochrome-f from the higher plant chloroplasts. The component exhibiting the 559 nm change has the properties of a cytochrome similar to cytochrome b<sub>3</sub>. Cytochrome b<sub>3</sub> is now presumed to have a role in cyclic II photophosphorylation.

Two of the mutant strains, ac-115 and ac-141, were found to lack the 559 cytochrome and light of photosystem I (at ~720 nm) induced the oxidation of only cytochrome 553. Mutant strain ac-206, which lacked the 553 cytochrome, exhibited only the PSII light (at ~680 nm) induced reduction of the 559 cytochrome. Mutant strain ac-21, which either lacked a component or had a component affected, exhibited absorbance changes attributable to both cytochromes. On illumination with PSII and PSI light, an indication of reduction of the 559 cytochrome and oxidation of cyt 553 were noticed in ac-21. These findings suggest that cytochrome 559 has been reduced by PSII and transferred electrons via an unknown or an affected component in ac-21 to the 553 cytochrome. This component is thus responsible for coupling the oxidation and reduction of the cytochromes mentioned above. Further, system I is suggested to cause the oxidation of the three components of the electron transport chain. The missing component in the electron carriers chain was subsequently discovered to be plastoquinone by Cramer *et al.*<sup>50</sup>.

Table 1—Photosynthetic Mutants Used in Studies on the Mechanism of Photosynthesis

Species and team of scientists	Mutant strain	Affected component
<i>Chlamydomonas reinhardtii</i> (Levine et al.)	ac-115, ac-141 ac-21 ac-206 ac-208 ac-80a ac-20 Ifd-2, Ifd-13, Ifd-15 Ifd-17, Ifd-27, Ifd-29	Cyt b 559, PQ M (an unidentified intersystem intermediate) Cyt c 553 PC P 700 RHDP Carboxylase Cyt b 559 (high potential)
<i>Chlamydomonas reinhardtii</i> (Sato et al.)	F-54	Photophosphorylation
<i>Chlamydomonas reinhardtii</i> (Togasaki et al.)	F-60	Phosphoribulosekinase
<i>Scenedesmus obliquus</i> (Bishop et al.)	Lip 10-2	Uniparental mutation affecting a specific partial reaction of photosynthesis, namely photophosphorylation.
<i>Scenedesmus obliquus</i> (Bishop et al.)	ScD <sub>3</sub> -a, ScD <sub>3</sub> -4 ScD <sub>3</sub> -5, ScD <sub>3</sub> -10 ScD <sub>3</sub> -11, ScD <sub>3</sub> -15 ScD <sub>3</sub> -40, ScD <sub>3</sub> -42 ScD <sub>3</sub> -67, ScD <sub>3</sub> -17 ScD <sub>3</sub> -18, ScD <sub>3</sub> -47 ScD <sub>3</sub> -8	Blocked PSII electron transport
<i>Scenedesmus obliquus</i> (Bishop et al.)	LF-1, LF-3 & LF-5	P700 Blocked on the oxidising side of PSII
<i>Oenothera hookeri</i>	ScD-26, ScD-50	Blocked PSI electron transport: diminished electron transport.
<i>Oenothera sneveolens</i> (Fork et al.)	I, Iy, Io-, IIy	Intersystem intermediates
<i>Synechococcus cedarorum</i> (Sherman et al.)	II Tsfl-1, 2, 3, 5, 7, 9, II and 12	Cyt f, P700 Categorised into three classes: (1) tsfl-1, 2: increased variable fluorescence; tsfl-1; a slight pigment mutations; (2) tsfl-3, 5: Abnormal morphologies; and (3) tsfl-7, 9, 11 and 12: High fluorescent, perturbed photosynthetic rates.

Levine and Gorman<sup>18</sup> indicated from the above experiments the presence of another b-type cytochrome having an absorption peak at 563 nm. However, its function is not yet clear<sup>1</sup>. It is presumed that this b-type cytochrome is associated with PSII and is responsible for cyclic photophosphorylation around PSI.

The ingenious and precise way of screening mutant strains used by Levine and German<sup>20</sup> also led to the conclusion that plastocyanin, a copper protein, is located between Cyt f and P700. They opined that a PC-less mutant ac-208 could still photoreduce but not photo-oxidise cyt f and the oxidation of cyt f would become possible only after an exogenous addition of PC. This was contested by Knaff and Arnon<sup>51</sup> who claimed that a PC-depleted preparation could yet photo-oxidize cytochrome f. Haehnel et al.<sup>52</sup> recently confirmed the position of PC as an immediate electron donor of P700 and studied in detail the functions of plastocyanin.

Six low fluorescence *Chlamydomonas reinhardtii* mutant strains, Ifd-2, Ifd-13, Ifd-15, Ifd-17, Ifd-27 and Ifd-29, which have been blocked on the electron donor

side of PSII were isolated and studied by Epel and Levine<sup>53</sup> and Epel et al<sup>54</sup>. Although these mutants are similar to normal *Chlamydomonas* in respect of total Chl content, Chl a:b ratio, the Chl a fluorescence excitation spectra, they do not photoevolve oxygen. Compared to the wild-type *Chlamydomonas*, these mutants have only one half of the high potential (ascorbate reducible) cyt b 559. According to Epel et al.<sup>54</sup>, this may suggest the existence of two distinct pools of the potential cyt b 559 which operate on the water splitting side of PSII.

#### (2) Mutations Inducing Loss of Photophosphorylation

In 1971, Sato et al.<sup>55</sup> reported that a mutant strain of *Chlamydomonas reinhardtii* F-54 was lacking in photophosphorylation capacity and possessing a non-labile  $\text{Ca}^{2+}$  dependent ATP-ase activity. Bennoun and Chua<sup>56</sup> reported that F-54 lacked three thylakoid membrane polypeptides visible normally in sodium dodecyl gel electrophoresis patterns and exhibited higher delayed luminescence yield compared to wild type. Further, the function of coupling factor ( $\text{CF}_1$ ) is

corroborated by the *Chlamydomonas reinhardtii* mutant F-54 which is unable to phosphorylate due to lack of both the 90A<sup>-</sup>CF<sub>1</sub> particles located on the outside of the thylakoid vesicles and the light activated Mg<sup>2+</sup> dependent ATPase<sup>57</sup>. In general, coupling factors are membrane bound particles and the Mg<sup>2+</sup> or Ca<sup>2+</sup> dependent ATPase are membrane bound soluble enzymes suggested to be responsible for photophosphorylation or for ATP formation in the chloroplasts. In 1971, Zilinsky *et al.*<sup>58</sup> selected arsenate resistant mutants of *R. capsulata* and among them found one strain with very high levels of the coupling factor per unit membrane or per bacteriochlorophyll compared to the normal strains. However, very little is being done with the enzymes obtained in this manner. Whereas point defects in the genome result in simple missing of components resulting in a block in the electron flow, extranuclear mutations such as in the plastome cause grosser abnormalities. Recently, Hudock *et al.*<sup>59</sup> reported a uniparentally inherited mutation affecting photophosphorylation in *Chlamydomonas reinhardtii* mutant lip 10-2. They observed that there is no detectable noncyclic photophosphorylation activity [using K<sub>3</sub>Fe(CN)<sub>6</sub>, MV as electron acceptors] in the mutant preparations, but the cyclic photophosphorylation activity was found at a rate 7% less than that in the case of the wild type. The equivalent cell preparations from wild type showed significant activity. Thus, lip 10-2 is capable of substantial photosynthetic electron transport activity, but it is incapable of photophosphorylation<sup>59</sup>. The very low rate of photosynthetic O<sub>2</sub> evolution in the mutant lip 10-2 which is less than 6% of that in the wild type is suggestive of an extremely low level of photophosphorylation of the same mutant. In agreement with the above findings, lip 10-2 was incapable of incorporating <sup>14</sup>C-acetate (a light dependent phenomenon) which is a measure of *in vivo* photophosphorylation. Usually, a majority of uniparental mutations involve antibiotic resistance or acetate autotrophy.

Nielson *et al.*<sup>60</sup> reported the effects of mutations in grana rich and grana deficient chloroplast mutants of barley in which photophosphorylation was affected. They reported that mutants having a high Chl a/b ratio catalysed 15-50 fold higher rates of ferricyanide photoreduction than the mutants having low Chl a/b ratios and 5-7 fold higher than in the wild type. Mutants catalysing PSI and PSII electron transport were tightly coupled and the rates of electron flow increased in the presence of methylamine, an uncoupler, and were able to generate a light dependent proton gradient, though differences in the rates of electron transport per mole/chlorophyll molecule were apparent.

### (3) Mutations Inducing Effects in the Photosynthesis Associated Properties

Mutants having electron paramagnetic resonance were first described by Bishop. Bishop<sup>43</sup> determined the EPR absorption characteristics of the two types of *Scenedesmus* mutants. Earlier it was understood that at least two distinct signals were induced upon illumination of chlorophyll by light. They have been characterized mainly on the basis of their kinetics of appearance and disappearances and 'g' values. Of the two signals, 'S' signal is slow and broad and is associated with reactions involved in the production of oxygen, while the 'R' signal is narrow and rapid decaying and is associated with an early photo-act in photosynthesis. Studies conducted by Bishop<sup>43</sup> using *Scenedesmus* mutants ScD<sub>3</sub>-8 and ScD<sub>3</sub>-40 confirmed the above conclusion. All the oxygen mutants having blocked oxygen evolution showed only a narrow rapidly decaying signal (R), while only a trace of 'S' signal was present. The kinetics of rise and decay of R signal were observed in the mutant ScD<sub>3</sub>-40 which revealed an almost instantaneous rise on turning on the light and an equally rapid decay on darkening. This is in contrast with the normal strain. Conversely, mutant ScD<sub>3</sub>-8, which is CO<sub>2</sub>-mutant, showed a prominent 'S' signal. These studies also revealed on a more careful examination that a component with an absorption peak at 700 nm was absent in the CO<sub>2</sub> mutant ScD<sub>3</sub>-8. It is suggested following these studies that P700 may be responsible for the 'R' signal, as it undergoes rapid oxidation and reduction during illumination.

Further usefulness of the mutant studies is associated with identifying the Mn-protein responsible for oxygen evolution. Recently, Spector and Winget<sup>61</sup> and James and Bishop<sup>62</sup> independently attempted to isolate the oxygen evolving enzyme. Using low fluorescent *Scenedesmus obliquus* mutants LF-1, LF-3 and LF-5, James and Bishop<sup>62</sup> concluded that 34 kdalton polypeptide of the wild type is probably associated with the Mn requiring protein of the water splitting complex of PSII. On the other hand, Winget and Spector<sup>61</sup> reported that two atoms of Mn bound to each 600 kdalton protein molecule get removed on tris alkaline washing and render the protein complex incapable of restoring O<sub>2</sub> evolution; this may be a part of the water oxidation enzyme system.

Photosynthetic characterization of mutants is not a simple task by itself. Sherman and Cunningham<sup>63</sup> reported a preliminary characterization of eight temperature sensitive high fluorescent *Synechococcus cedrorum* mutants tsf-1, 2, 3, 5, 7, 9, 11 and 12, which can be placed under three categories based primarily on fluorescence induction kinetics. The details of these mutants are furnished in Table 1. All the mutants have

modified protein patterns when grown at 40°C and they thus appeared to be nonidentical mutants. The protein patterns of these mutations indicate that they are distinct and can affect numerous functions within the membrane. It is obvious that fluorescent aberrations can be caused by perturbing a variety of functions. Since very few photosynthetic membrane proteins have been identified, these mutations provide an opportunity to probe the structure-function relationship in depth.

While working on the isolation of photosystem II fragments of *S. cedrorum*, Sherman and Neumann<sup>64</sup> discovered a protein (mol. wt 47,000) which is a component of PSII. A protein of this molecular weight is defective in one of the temperature sensitive fluorescent mutants, tsf-11, characterized recently by Sherman and Cunningham<sup>63</sup> who are now trying to ascertain whether these proteins are identical or not. Earlier, Chua and Bennoun<sup>65</sup> reported that the F-34 mutant of *C. reinhardtii* strain lacked a protein of similar molecular weight (47,000) which is required for PSII activity.

One of the significant developments of research on biochemistry of photosynthesis is to induce resistance or insensitivity of DCMU or its analogues into photosynthetic reactions of chloroplasts, particularly on the acceptor side of PSII.

Some such DCMU resistant strains of the green algae *Chlamydomonas* and *Euglena* were described by McBride *et al.*<sup>66</sup>, Bouges-Bocuet<sup>67</sup> and Laval Martin and Galvayrae<sup>68, 69</sup> independently. Similarly, triazine resistant species of *Brassica*<sup>70</sup>, *Amaranthus*<sup>71</sup>, *Chenopodium*<sup>72</sup> and *Senacio*<sup>73</sup> were reported in which triazine resistance is shown to be due to an alteration of the thylakoid membrane; as a consequence, the binding affinity for triazine is significantly lower in the mutant, whereas that of DCMU or phenol inhibitor remains the same. Therefore, it is presumed that the binding area of the B-protein contains a common binding area for all inhibitors, but with specific binding sites for DCMU, triazine and phenols, the resistant species lose one or more of such specific sites.

#### Chemical Inhibitor Techniques

In the light of the significance gained by inhibitor studies, the application of artificial electron acceptors and donors has obviously increased manifold. Apart from the natural electron acceptor NADP<sup>+</sup>, viologen dyes, such as methyl viologen (MV), benzyl viologen (BV), anthraquinone-2-sulphonate and diaminodurene (DAD) are extensively employed as artificial electron acceptors at a position preceding Fd (see Figs. 1 and 2 for their positions and structures). Ferricyanide and dichlorophenol indophenol are also electron acceptors and accept electrons mostly between the two

photosystems and sometimes accept electrons from PSI when both PSI and PSII are linked.

Under natural conditions, water acts as a natural electron donor in photosynthesis. In addition to water, a group of electron donors which may compete or substitute for water are widely employed now. Ascorbate, hydroquinone, hydroxylamine, phenylenediamine, diphenylcarbazide and manganous ions are among the donors commonly employed. Also, a number of artificial electron donors have been applied in between two photosystems. A few among them are ascorbate + DCPIP, ascorbate + TMPD, and ascorbate + DAD. Mention may be made here of 5-diphenylcarbazone which in PSI sensitized reactions gets oxidized and reduced simultaneously and acts both as an electron donor and acceptor.

At present, the available inhibitors can be grouped according to five areas of attack on the photosynthesis electron flow: Inhibitors of the acceptor and donor sites of PSI and PSII and PQ antagonists<sup>2</sup>. A bewildering variety of inhibitors have been applied to unravel the mechanisms involved in photosynthesis during the last two decades.

Apart from the well known inhibitors, such as DBMIB and DCMU, several other inhibitors such as  $\alpha$ -benzyl- $\alpha$ -bromomalodonitrile<sup>27</sup>, chelators<sup>29</sup> (butanedione derivatives), chaotropes<sup>30</sup>, polylysine<sup>35</sup>, ethyleneglycol (bis-aminoethyl ether), N, N, N', N'-tetraacetic acid<sup>36</sup>,  $\beta$ -naphthyl oligophosphates<sup>37</sup>, diphenyl ether herbicides<sup>38</sup>, pyrone derivatives<sup>39</sup>, 2-sec-butyl-4-5-dinitrophenol (dinoseb)<sup>41</sup>, tetraniitromethane<sup>42</sup> and linolenic acid<sup>74</sup> have found significant use in inhibitor studies. In addition, the effects of copper<sup>75, 76</sup>, lead and cadmium<sup>77-80</sup> ions on electron flow and PSII in particular were investigated thoroughly. Wong and Govindjee<sup>28</sup> reported the inhibition of the reaction centre P700 of PSI on the addition of lead salts. Govindjee and Rabinowitch<sup>81</sup> reported the inhibition of photosynthesis through the application of extreme red light in certain algae. Govindjee *et al.*<sup>82</sup> reported that the reoxidation of secondary electron acceptor of PSII was inhibited in bicarbonate depleted chloroplasts. A few devious methods are highlighted to emphasize the versatility and extensive applicability of inhibition techniques.

Inhibitor studies are closely dependent on artificial donor or acceptor systems, on the mode of action of inhibitors and on their structural relationship to the biological activity.

#### (I) Inhibition on the Electron Donor Side of PSII

A few inhibitors and several treatments which include gentle heating of chloroplasts at 55°C for 5-6 min, washing with 0.8 M tris buffer (pH 8) for 15 min and hydroxylamine treatment inhibit the electron donating side of PSII.

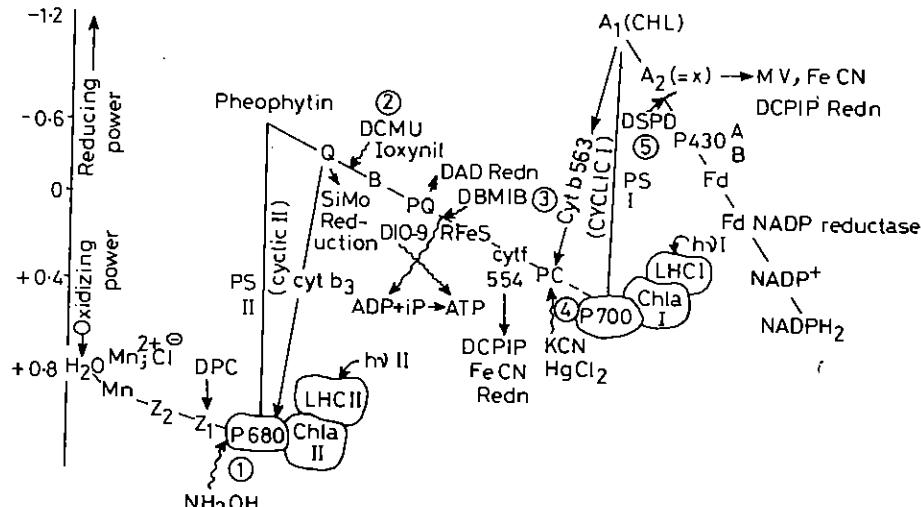
Hydroxylamine and other amines under some peculiar conditions inhibit the  $O_2$  evolving reaction. Since hydroxylamine also acts as an artificial electron donor beyond its site of inhibition, it may not be an efficient inhibitor. Pretreatment of the chloroplasts with high concentrations of tris is probably the most commonly employed procedure for the inactivation of oxygen evolving systems in the presence of light<sup>83</sup>. Consequent upon tris treatment, manganese is released from the membrane into the inside thylakoid space and remains bound, indicating localization of the water splitting system towards the interior space of the membrane.

The combined effect of external pH (9.0-9.3) and internal pH in the presence of methylamine results in

the inhibition of electron flow on the electron donating side of PSII. Inhibition by internal pH has recently been found to be due to a specific inactivation of O<sub>2</sub> evolution, because donor systems for PSII remain intact at high internal pH<sup>84-86</sup>. It was subsequently understood that it was the S<sub>2</sub> state of oxygen evolution which was sensitive to internal pH<sup>87</sup>.

Another way of causing inhibition on the electron donating side of PSII is synthesis and application of antibodies. Braun and Govindjee<sup>88, 89</sup> and several other workers used antibodies to inhibit electron flow at the donor side of PSII.

As mentioned earlier, inhibition by cadmium salts<sup>78-80</sup> is more specific and 1-10  $\mu\text{M}$  cadmium nitrate seemed to affect the donor side of PSII. Rita *et al.*



**NH<sub>2</sub>OH**

Fig. 1—Photosynthetic electron pathway—Photosynthesis is driven by the concerted action of two photosystems: Photosystem II and photosystem I. Both photosystems consist of a number of accessory pigments, carotenoids, phycobilins and chlorophyll b molecules which impart sufficient energy to the reaction centres of each photosystem (Duyens *et al.*). The reaction centres of PSII and PSI are coined as P680 and P700 respectively (680 and 700 stand for their wavelengths in the red region and far red region of the visible spectrum). To attain the maximum efficiency of photosynthesis, both systems are to function simultaneously (Emerson's red drop and enhancement effect) regardless of the kind of photophosphorylation—non-cyclic or cyclic (cyclic I and cyclic II). To obtain a single molecule of oxygen during photosynthesis, four electrons and four protons must pass through the entire system, but one at a time. As a result of this, the photoreducing equivalents ATP (adenosine triphosphate) and NADPH (nicotinamide adenosine dinucleotide diphosphate reduced form) are produced. Both ATP and NADPH together provide energy for carbon dioxide fixation in the green leaves during the dark phase of photosynthesis. The entire process mentioned above is not as simple as literally presented here. The excited reaction centre of PSII, P680 ejects out an electron which reduces the immediate primary electron acceptor, Q, of the Photosystem II. Q stands for the "quenching" of fluorescence. This sparks off a chain reaction in the entire system and the excited electron passes through a number of electron carriers in a redox reaction sequence. The oxidized P680 draws an electron from an unknown donor designated 'Z' and returns to ground state. After 'Z' has given up four such electrons, it oxidizes water and recovers the same from water. Whereas electron flow from water to carbon dioxide proceeds against an electron chemical gradient of 1.2 V, the two photosystems require 8-10 quanta of energy for the inflow of 4 electrons (1 electron = ~2 quanta). The portion of the photosynthetic electron transport chain that carries electrons from water to P680 is known as the 'oxidizing side'. The 'reducing side' of PSII is the portion of electron transport chain between PSII and its electron acceptor Q. Cyt b 559 low and high potential, PQ, Cyt f, Reiska Fes-Centre, and PC are the constituent electron carriers of the down Hill pathway (Hill and Bendall model). Plastocyanin (PC) injects electron into PSI, designated P700, which is in oxidized state following ejection of an electron from it to the primary acceptor A1 (chlorophyll). The electron thus passes through A<sub>2</sub> (X), ferredoxin (FD) and the enzyme ferredoxin-NADP reductase (R) to NADP<sup>+</sup> which would be reduced to NADPH. Uncertainty prevails with respect to the number of ATP molecules and the site of generation of the second ATP molecule. Usually, three ATP molecules and NADPH are required for CO<sub>2</sub> fixation (Levine *et al.*). As regards the site of inhibition of various inhibitors, five significant points of inhibition are present here. Dio-9, an antibiotic, inhibits energy transfer between PQ and Cyt f, where dibromothymoquinone (DBMIB) exactly inhibits the electron flow. Dichlorophenyl dimethyl urea (DCMU) inhibits between (Q) and (B). The site of inhibition by potassium cyanide and mercuric chloride is located at PC. On the PSII oxidizing side, hydroxylamine acts as an inhibitor at the site of the water splitting mechanism. On the PSI side, disalicylidene propane diamine (DSPD) inhibits at the Fd-level and it also interacts with Cu, which is suggested to be involved on the oxidizing side of PSII. For convenience of understanding, several important artificial electron donors and acceptors and their sites of action are presented here.

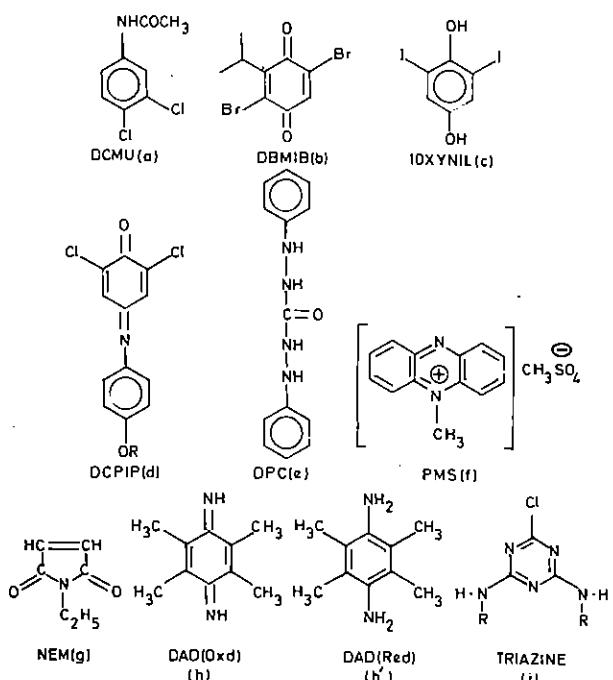


Fig. 2—Structures of some important inhibitors, artificial electron acceptors and donors [(a)3-(3, 4-Dichlorophenyl)-1, 1-dimethyl urea, (b) 2, 5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, (c) ioxynil, (d) 2, 6-dichlorophenolindophenol, (e) diphenylcarbazide, (f) phenazinemethosulphate, (g) N-ethylmaleimide, (h) diaminodurene (both oxidized and reduced forms) and (i) triazine]

al.<sup>36</sup> recently pointed out two sites of inhibition by ethyleneglycol (*bis*-aminoethyl ether) N, N, N', N'-tetra-acetic acid, a calcium chelator; of these, one site (site II) of inhibition is localized between Mn<sup>2+</sup> site and PQ pool on the electron donating side of PSII. Rita and Crane<sup>29</sup> studied the organization of electron transport on the water donating side of PSII in spinach chloroplasts using chelators like 4, 4, 4-trifluoro-1-(2-thienyl)-1-1-butanedione. The partial reactions used included H<sub>2</sub>O → MV, H<sub>2</sub>O → silicomolybdic acid, H<sub>2</sub>O → FeCN and H<sub>2</sub>O → DBMIB. Inhibitions common to all pathways presumably affect the Mn or water oxidation site in PSII.

Younis and Mohanty<sup>41</sup> by using a herbicide 2-sec-butyl-4,6-dinitrophenol (dinoseb) have drawn attention to a contrast between water supported DCPIP photoreduction and DPC supported DCPIP photoreduction. They concluded that the inhibition of electron flow by dinoseb is located at a site close to the PSII reaction centre in uncoupled chloroplasts. Further, dinoseb is reported to have inhibited photo-phosphorylation and Mg<sup>2+</sup> dependent ATP-ase activity. Similarly, Sane and Johanningmeier<sup>41</sup> studied the effects of tetraniromethane on the electron donating side of PSII. At low concentrations (10 μM), tetraniromethane inhibits non-cyclic electron transport, primarily due to the tetraniromethane

interference with the donor side of PSII prior to the donation of electrons to PSII. These workers suggested that a functional —SH group is essential for a protein which mediates electron transport between the water splitting complex and P680 of PSII.

#### (2) Inhibition on the Electron Acceptor Side of PSII

DCMU and its analogues, such as anilides, biscarbamates, uracils, triazines, triazinones, pyridazinones, pyrimidinones and trifluoro-benzimidazoles are known for effective inhibition brought about by them at the reducing side of photosystem II after the primary acceptor Q; they possess a common structural component, an SP<sup>2</sup> hybrid-C-NH, which is considered to be essential for causing inhibition and for binding to the membrane.

DCMU at 1 μM concentration inhibits the electron flow between Q and B (Fig. 1)<sup>23</sup> and the Hill reactions which include the main plastoquinone pool whether driven by both photosystems or by photosystem II alone become DCMU sensitive. However, photoreductions by PSI with the mediation of artificial electron donors as well as cyclic electron flow involving PSI become DCMU insensitive. Reversal of electron flow from PQ to Q is also DCMU sensitive, suggesting localization of the inhibition site strongly in between Q and B. Although the functional site of inhibition is localized between Q and PQ, its mode of action still remains a mystery. Possibly, DCMU is binding to a polypeptide of a protein (B-protein) which covers the reducing side of PSII and is believed to be a part of PSII protein complex and catalyzes B(R) as a prosthetic group. The part of the B-protein of PSII with B(R) as a prosthetic group, which shields the primary electron acceptor Q, carries binding sites for inhibitors like DCMU, triazine and phenols. It is also postulated that the binding area is common to all these inhibitors and overlapping is unavoidable. It is opined that the inhibitor induces a conformational change in the B-protein, which, in turn, disturbs its functioning in catalyzing electron flow from Q to PQ<sup>90, 91</sup>.

Phenols like ioxynil, nitrophenols and dinitrophenols with alkyl substitutions have been identified as powerful electron flow inhibitors<sup>39, 91-93</sup> and have been known for causing inhibition of electron flow in chloroplasts in a way akin to DCMU inhibition. Though these compounds do not have the basic chemical structure that DCMU has for inhibition, the comparison and relation of structure to the functions of approximately 40 substituted alkyl nitrophenols revealed that the inhibitory potency is proportional to the size of the substituent and not to the pK of the —OH group or the lipophilicity of the compound.

Another group of compounds, "azaphenanthrenes", represented by O-phenanthroline (1, 10-diazaphen-

anthrene) were prepared by Oettemeier and Greve<sup>94</sup> and were shown to inhibit electron flow at the reducing side of PSII after the primary acceptor Q. O-Phenanthroline at 100  $\mu\text{M}$  concentration inhibits electron flow in a manner responsible for inhibition, although Rita and Crane<sup>29</sup> and Beyring *et al.*<sup>95</sup> contended that the chelating potency of iron complex in these inhibitors is responsible for inhibition.

### (3) Inhibition on the Electron Acceptor Side of PSI

There is sufficient evidence to show that disulphodisalicylidene propane diamine or sulpho-DSPD is a representative of a group of metal chelating compounds and inhibits the photosynthetic electron flow on the acceptor side of PSI, presumably the ferredoxin reaction<sup>96</sup>. It might actually attack ferridoxin itself<sup>97</sup>. Because of its high hydrophilicity due to the sulpho group, the inhibition is taken as evidence for the accessibility of the endogenous acceptor side of PSI in the thylakoid membrane. DSPD also inhibits Hill reactions, but does not attack artificial electron acceptor systems for PSI. The sulpho group of DSPD is a disadvantage, as it cannot be used due to its hydrophilicity in intact systems. Therefore, the parent compound DSPD devoid of sulpho group is ordinarily used, as it acts in the same way as sulpho DSPD. However, divergent views have been expressed regarding the validity of the concept that both DSPD and sulpho DSPD act similarly.

### (4) Inhibition on the Electron Donor Side of PSI

It is generally accepted that plastocyanin is the immediate electron donor to PSI in intact chloroplasts and the inhibitors of plastocyanin delink PSI from PSII, resulting in disruption of non-cyclic photophosphorylation. However, the activity of PSII, including electron flow from water to an artificial lipophilic acceptor for PSII, remains uninterrupted. Cyt f photooxidation is inhibited due to plastocyanin inhibition, but the oxidation of P700 of PSI is not inhibited. Kiminura and Katoh<sup>98-100</sup> first reported the effect of HgCl<sub>2</sub> on electron flow and localized its inhibition which takes place at a concentration stoichiometric to chlorophyll concentration. It is estimated to be 10  $\mu\text{M}$  to 10  $\mu\text{g}$  chlorophyll. At higher concentrations of HgCl<sub>2</sub>, PSII activity is also disturbed<sup>101</sup>.

Recently, a combined treatment with HgCl<sub>2</sub> and KCN was recommended by Yocom and Guikema<sup>102</sup> for PC inhibition. They reported that 10  $\mu\text{M}$  HgCl<sub>2</sub> + 50  $\mu\text{M}$  KCN (at pH 8.0 and 4°C for 90 min) combination gives reliable and reproducible results on the coupling of PSII and of photophosphorylation driven by PSII. HgCl<sub>2</sub> at minimal concentration (0.1  $\mu\text{M}/\mu\text{g}$  chlorophyll) does not

interrupt coupling of PSII, but coupling of PSI electron flow is fully inhibited<sup>103, 104</sup>.

At low salt concentrations and maximum swelling of chloroplasts, polylysine is known to inhibit electron flow on the donor side of PSI<sup>23</sup>. PSI activity is inhibited by polylysine at 1 mg or 0.1 mg histone concentration<sup>105</sup>. The binding of polylysine to PC apparently prevents the reduction of plastocyanin by Cyt f<sup>35</sup>. Histone application inhibits PSI activity in chloroplasts from a variety of higher plants, but it is not inhibitory to PSI in a cell-free preparation of algae<sup>23</sup>. Synthetic polylysines inhibit in similar fashion as histones. Studies using HgCl<sub>2</sub> and KCN at different concentrations revealed two sites of coupling located in between two photosystems<sup>103, 104, 106</sup>. Using 30 mM KCN along with a trace of ferricyanide to keep plastocyanin oxidized, Ouitrakul and Izawa<sup>107</sup> treated chloroplasts for 30-70 min. This indicated the coupling of KCN insensitive PSII, Hill reactions and thereby the existence of a second coupling site. Wong and Govindjee<sup>28</sup> reported the direct inhibition of the reaction centre P700 of PSI in isolated maize chloroplasts, when the lead salts were added.

### (5) Plastoquinone Antagonists and Inhibition on the Oxidizing Side of PQ

With the discovery of plastoquinone antagonist DBMIB<sup>15</sup> and the application of DBMIB to inhibit electron flow at the oxidizing side of PQ, a new vista has been thrown open. Warburg and Luttgens<sup>3</sup> had first reported in 1944 the role of *p*-benzoquinone as an acceptor of Hill reaction. Also, Govindjee *et al.*<sup>106</sup> studied the implications of *p*-benzoquinone photoreduction in chlorella cells and documented the second Emerson effect based on the action spectra in chlorella.

A comparison of quinones as Hill acceptors during 1960-1963 showed that the photoreduction of all quinoid compounds is coupled to ATP formation. A number of pairs of quinoid compounds having similar structures and chemical properties but with different lipid solubilities such as phenazine/sulphophenazine, naphthoquinone/naphthoquinone sulphate; indophenol/sulphoindophenol, in the oxidized form, are established as the best acceptors of electrons in the photosynthetic electron transport chain in Hill reactions. Some quinoid compounds having redox potential lower than 0 volt are considered as cofactors in cyclic photophosphorylation. *p*-Benzoquinones, which have redox potentials more positive than 0 volt, are used for titration of the redox potential of the acceptor side of PSII in mutant strains of algae lacking in PSI activity<sup>108, 109</sup>. Most of the lipophilic compounds in the reduced form can donate electrons to PSI, when electron flow from PSII is blocked by the inhibitor<sup>110</sup>. Therefore, it is asserted that the oxidizing

site of PSII and the electron donors for PSI (PC) are located inside the lipid barrier of the inner chloroplast membrane.

The sensitivity of a photosynthetic system to DBMIB is today taken as evidence for plastoquinone participation in the system. There is no influence of DBMIB on the Hill reactions by PSII, but there is inhibition of Cyt f<sup>111,112</sup>, plastocyanin and P700<sup>113,114</sup> reduction and their oxidation remains unaffected. Haehnel<sup>114</sup> noted that DBMIB did not interfere with the reduction of PQ by PSII, but pointed out the inhibition of its re-oxidation by PSI. The influence of DBMIB on the fluorescence induction curve<sup>115</sup> as well as oxygen liberation indicated that DBMIB does not disconnect the secondary plastoquinone pool from PSII. The number of DBMIB molecules required for inhibition is calculated to be about one per 300 chlorophylls or per one electron transport chain<sup>114</sup>. DBMIB also inhibits photosynthetic NADP reduction with water as an electron donor. At concentrations of  $8.10^{-7} M$  and  $2.10^{-6} M$ , the inhibition is 50 and 100% respectively. The photoreduction of NADP at the expense of an artificial electron donor, DAD/ascorbate, however, is not affected even at high concentrations ( $10^{-5} M$ ) of the inhibitor. Whereas 100% inhibition of electron transport from water to anthraquinone, methyl viologen or NADP is obtained at  $2.10^{-6} M$ , the reduction of ferricyanide is inhibited to only about 60%, whether the system is coupled or uncoupled.

It is tempting to speculate that DBMIB may not be inhibiting PQ as such, but rather binding to the site on a protein carrier-complex like Rieske FeS Center at which plastoquinone is oxidized. In fact, the final rate of electron transport in anthraquinone, methyl viologen and NADP mediated systems remains the same in the presence of  $10^{-6} M$  or more DBMIB and whether coupled, not coupled or uncoupled. Further, the reduced form of DBMIB shows the same effect as the oxidized form. The different criteria for inhibition by DBMIB distinguish the inhibitor from the known inhibitors of photosystem II. Most of the substituted benzoquinones analogous to DBMIB and also certain alkylated dinitroanilines and diphenyl ethers have shown inhibition similar to that of DBMIB. Also, bathophenanthroline and valinomycin (an antibiotic) are shown to have inhibited the electron flow between PQ and PC.

A TMPD bypass is suggested by Trebst and Reimer<sup>116-118</sup> and Selman<sup>119</sup> as an alternative electron pathway from the site of inhibition of PQ to plastocyanin in the presence of DBMIB. The TMPD bypass is coupled to ATP formation with a very high P/O<sub>2</sub> ratio; particularly the TMPD bypass has proved highly helpful in locating other inhibitor sites<sup>116</sup>.

However, TMPD bypass is not a reversal of inhibition, because the inhibited step remains inoperative. But thiol compounds such as mercaptoethanol, DTT and bovine serum albumin would reverse the DBMIB inhibition. Bovine serum albumin at 1 mg level reverses the inhibition of DBMIB independent of its redox state<sup>120,121</sup>. Thiols usually inactivate DBMIB, but after preillumination when DBMIB falls into reduced state, and on the addition of further amounts of thiols there is reversal of inhibition. Guikema and Yocom<sup>32</sup> registered evidence for two sites of inhibition by DBMIB. They noticed that DBMIB at  $1 \mu M$  concentration imposes maximal inhibition in the case of reactions involving electron transport from water to ferricyanide and at  $\mu M$  concentration inhibit electron transport reactions catalyzed by PSII in the presence of *p*-phenylenediamines or *p*-benzoquinones. They observed this in both untreated and KCN/Hg inhibited chloroplasts. Whereas the reversal of inhibition at the first site (high affinity site) requires high thiol/inhibitor ratios and incubation periods up to 3 min, the second site (low affinity site) requires thiol/inhibitor ratio of 1 and incubation periods as short as 55 sec. Guikema and Yocom<sup>32</sup> also ruled out the possibility of TMPD bypass.

2, 4-Dinitrophenothers of bromonitrothymol, iodonitrothymol and ioxynil synthesized by Trebst *et al.*<sup>91,123</sup> were shown to have an inhibition mode similar to that of DBMIB. Methyl, 2,4-(4-nitrophenoxy)-4(trifluoromethyl benzene) synthesized by Wayne *et al.*<sup>38</sup> were shown to inhibit linear electron transport from water to ferricyanide or methyl viologen. In diphenyl ether treated chloroplasts, the half time for the dark reduction of Cyt f is increased 5-10 fold. Cyclic electron flow catalyzed by phenazine, methosulphate or DAD is resistant to inhibition by nitrofluorofen.

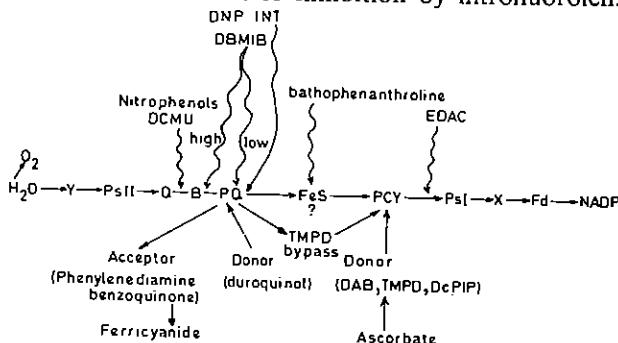


Fig. 3—A TMPD bypass as suggested by Trebst *et al.*, with inhibitors like DBMIB operating on the plastoquinone oxidation site. Bathophenanthroline, a copper and iron chelating agent, has inhibition pattern similar to that of DBMIB at a concentration of  $100 \mu M$  and is supposedly inhibiting electron flow between PQ and Cyt f. By virtue of its iron chelating capacity, it would act on a non-heme iron protein functioning between PQ and PC. The two sites of inhibition, at low and high-concentrations of DBMIB, are also shown in the figure. A catalytic amount of TMPD can bypass the inhibition of bathophenanthroline.

Therefore, Wayne *et al.*<sup>38</sup> concluded that the site of inhibition for diphenyl ethers may be between the two photosystems in the PQ-Cyt f region.

Although DBMIB is regarded as the best inhibitor, it has certain weak points. Being a quinone, it is a redox compound and as such acts as an electron acceptor for PSII and as a mediator for electron flow from PSII to ferricyanide<sup>122</sup>. Also, due to their quinoid character nucleophiles, particularly -SH compounds, quickly inactivate DBMIB. It is, therefore, desirable to design a DBMIB-like inhibitor such as pyrones<sup>39</sup> without redox properties.

#### (6) Inhibition of Photophosphorylation

Electron transport is coupled to photophosphorylation. No ATP is made unless electron flow occurs and vice versa. Isolated chloroplasts are generally found to be not perfectly coupled and a basal electron transport occurs at rates between one half and one fourth those seen on adding ADP and P<sub>i</sub> with a control ratio of 2 to 3.5<sup>123</sup>. A violation of this occurs in the presence of chemical uncouplers, such as methylamine and ammonium chloride. Most recent reports on the inhibition of photophosphorylation are of Shinohara and Hidehero<sup>37</sup>, Younis and Mohanty<sup>41</sup>, and Samuelsson and Gunnar Oquist<sup>124</sup>. Earlier, McCarty and coworkers<sup>24</sup> reported the N-ethyl maleimide inhibition of photophosphorylation in which N-ethyl maleimide interfered with one of the  $\gamma$ -subunits of Coupling Factor 1. Recently, McCarty *et al.*<sup>25</sup> also interpreted the difference of action of mono-functional maleimides and bi-functional maleimides in linking up with the  $\gamma$ -sub units of CF1. Shinohara and Hidehero<sup>37</sup> reported that  $\beta$ -naphthyl oligophosphates inhibit the ATP-ase activity of chloroplasts, CF1 and light triggered ATP-ase activity of isolated chloroplasts.  $\beta$ -Naphthyl monophosphate acts noncompetitively. At concentrations equal to that of ADP or ATP,  $\beta$ -naphthyl di, tri and tetraphosphate inhibit three reactions in the order; ATP-ase of isolated CF1 > photophosphorylation > light triggered ATP-ase chloroplasts. The effect of monophosphate is apparently on pi site and that the di, tri and tetraphosphate on the adenine dinucleotide site(s) on the active centre CF1.

Younis and Mohanty<sup>41</sup> studied the effects of 2-sec-butyl, 4,5-dinitrophenol (dinoseb) on cyclic and non-cyclic photophosphorylation. They observed inhibition of both cyclic and non-cyclic electron flow and Mg-ATP-ase activity by dinoseb, which is suggestive of the fact that dinoseb is also an energy transfer inhibitor. The fact that the rate of non-cyclic photophosphorylation dropped to less than one third of its control value at 10  $\mu$ M concentration and became insignificant at 20  $\mu$ M concentration indicates that the

effect of dinoseb is greater on non-cyclic photophosphorylation than the rest of the other activities. Further, Younis and Mohanty<sup>41</sup> observed a strange kind of interaction between dinoseb with Mg<sup>2+</sup> ATP-ase activity and dinoseb which Ca<sup>2+</sup> ATP-ase activity. The most important observation is that dinoseb has been proved to be an energy transfer inhibitor and not an uncoupler; this had gone unnoticed earlier. Samuelson and Gunner<sup>124</sup> showed that Cu<sup>2+</sup> acts as an uncoupler of photophosphorylation in spinach chloroplasts at low concentrations. In the presence of ascorbate, the addition of Cu<sup>2+</sup> caused rapid inactivation of the PSI reaction from ascorbate/DCPIP to NADP.

#### Conclusion

The photosynthesis mechanism can be explained through inhibitor studies which can be broadly grouped into two classes: Mutagenic inhibitor studies and chemical inhibitor techniques. The recent work in this direction is reviewed. In spite of enormous research activity, mystery still surrounds certain areas of research in photosynthesis. As a result of decades of fruitful research, a scientific picture of photosynthetic electron flow has ultimately emerged. What provoke the scientists engaged in research on photosynthesis are the lurking electron carriers, such as cytochromes, Cyt b 559 (low and high potential) and Cyt b 563, located between two photosystems (PSII and PSI). Further, the roles of Mn in water splitting complex and Mg in energy coupling are posing a challenge. To develop an unambiguous and indubitable picture of photosynthetic electron flow, scientists have resorted to inhibiting electron flow either by mutagenic techniques or by the production of different types of chemical inhibitors. Structural relationships and the functional groups responsible for specific inhibition sites are yet to be established. Research is being continued and the day when the whole mystery of photosynthesis will be unravelled is supposedly not far off.

#### Summary

Efforts have been made to inhibit the photosynthetic electron transport at various crucial points of electron passage. Beginning with inhibitors like antimycin A, DCMU, DBMIB, chaotrope, chelators and dinitrophenyl ethers up to halogenated or hydroxylated benzoquinones, inhibitors have been studied extensively. Similarly, a number of mutants have been developed to characterize the missing electron carriers and components. These studies have greatly helped identify the components, particles and several functional areas of thylakoid membranes. Attempts have also been made to correlate the biologically active

groups of inhibitors with the functional components of thylakoids.

### Acknowledgement

The author's gratitude is due to Profs. Govindjee (Dept. of Biophys., Physiol., Bot., Univ. of Ill., Urbana, USA), Charles F Yocom (Dept. Biol. and Chem., Univ. of Michigan, USA), Achim Trebst (Ruhr-Universität Bochum, West Germany), Norman I Bishop (Oregon State Univ., USA) and Niels C Nielson (Indiana University, Indiana, USA) for sparing their precious preprints and reprints material for the preparation of this review. He is also thankful to Shri D. Ram Mohan, K. Prakashbabu and Shri K. Sreenivasa Rao for their invaluable technical assistance. He acknowledges his indebtedness to Profs. Govindjee, Prasanna Mohanty (JNU, New Delhi) and Dr. P.V. Sane (B.A.R.C., Bombay) for their criticism of the manuscript.

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